CLII. ON THE TECHNIQUE OF TESTING FOR THE PRESENCE OF VITAMIN A.

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The technique that has for some time past been employed in this laboratory for testing for the presence of vitamin A in foodstuffs, or fractions isolated during our efforts to determine the nature of that substance, has been described in previous publications [Drummond and Coward, 1920; Drummond and Watson, 1922, 1]. Until quite recently we placed considerable trust in this method, which, in broad detail, consisted of feeding young rats on an artificial ration purified as far as possible from fat-soluble vitamins until the deprivation resulted in a cessation of growth. The response in growth that followed the administration of the foodstuff, or other substance, was then interpreted as an indication of the amount of vitamin A that had been supplied. Our belief that this test, standardised as carefully as possible, yielded trustworthy results, was not disturbed when it was clearly demonstrated, first by McCollum and his co-workers, that the vitamin A is a substance distinct from the antirachitic factor, now somewhat generally referred to as vitamin D, because there was then no evidence to show that the latter substance might interfere with the test.

Steenbock and his colleagues have, however, by a very careful series of researches demonstrated that the cessation of growth that is exhibited by animals maintained on the more or less standard type of basal diet employed in testing for vitamin A may not always be due to a deficiency of that substance, but that in some cases it may be caused by an inadequate supply of vitamin D.

Recognising the importance of their observations, Steenbock, Nelson and Black [1924] devised a modification of the technique usually employed in testing for vitamin A, which consisted of supplying the antirachitic factor throughout the experiment either by a supplement of aerated cod-liver oil—it having been shown by McCollum, Simmonds, Becker and Shipley [1922] that the vitamin A in that oil is destroyed by 12–28 hours' oxidation by a current of air at 100°, whereas its antirachitic action survives—or by regular exposure of the test animals to radiations, from a suitable source, such as a

quartz mercury-vapour lamp, which are now known to endow certain substances with antirachitic power. On the whole, they prefer the latter alternative, and propose to maintain the animals on their ordinary basal diet of purified caseinogen, agar, yeast, salt mixture and dextrinised starch, but to guard against the incidence of disturbances arising from a shortage of the antirachitic substance by exposing the animals to the radiations of a quartz mercury-vapour lamp for a short period, say 10 mins. every day. In their experiments the control rats receiving no irradiation showed some growth, with incidence of ophthalmia in from four to six weeks. Those which received the same food mixture, but in addition were exposed to the radiations every day, grew considerably more, but showed signs of ophthalmia at about the same time as the controls. The present paper reports results which in essentials confirm those to which we have just referred, and we record them, in the first place, because they were arrived at from a somewhat different line of approach to the problem, and in the second, because we believe that our modification of the usual technique is slightly superior to that proposed by the investigators we have mentioned.

We were led to this subject by an investigation, recently reported by Drummond, Rosenheim and Coward [1925], during which it was found that cholesterol of a fairly high degree of purity[1] after exposure to the radiations of a quartz mercury-vapour lamp promotes a resumption of growth when administered to rats which have ceased to grow on the standard diet deficient in vitamin A.

The fact that cholesterol treated in this manner is also endowed with marked potency as an antirachitic has now been demonstrated with certainty [Hess, Weinstock and Helman, 1925, 1, 2; Hess and Weinstock, 1925; Steenbock and Black, 1925; Rosenheim and Webster, 1925].

At first we were inclined to believe, from the very prompt recovery which our animals showed in growth, that the radiation had also effected either a synthesis of vitamin A, or of some substance possessing similar physiological action. On the other hand, doubts were raised by two facts. In the first place the irradiated cholesterol, although it gave colour reactions differing markedly from those of the untreated compound[2], failed to give the colour tests that have been studied in this laboratory [Drummond and Watson,

[1] The purity of samples of cholesterol is an important matter in work of this type, where a minute trace of impurity may well be the precursor of the active substances formed during exposure to short wave-length radiations. Dr O. Rosenheim has given us the benefit of his wide experience of cholesterol and related compounds, and has emphasised the necessity of at least two saponifications with repeated crystallisations from more than one solvent before anything approaching pure cholesterol can be obtained. The product employed by us was three times subjected to hydrolysis with boiling alcoholic potash, with many intermediate and subsequent crystallisations, totalling 27 in all, alcohol, ether and light petroleum being used as solvents. It melted at 148·8° (uncorr.). Melting points as high as 150° have been recorded, and it is doubtful whether a product melting much below 149° can be regarded as satisfactory.

[2] The colour reactions of irradiated cholesterol, and their significance, will be the subject of a later communication by Dr O. Rosenheim.

1922, 2; Rosenheim and Drummond, 1925], which we are strongly inclined to believe are given by the substance known as vitamin A. Secondly, the response in growth that in most cases followed the administration of irradiated cholesterol was irregular, and could not be correlated in a quantitative manner with the amount of the supplement.

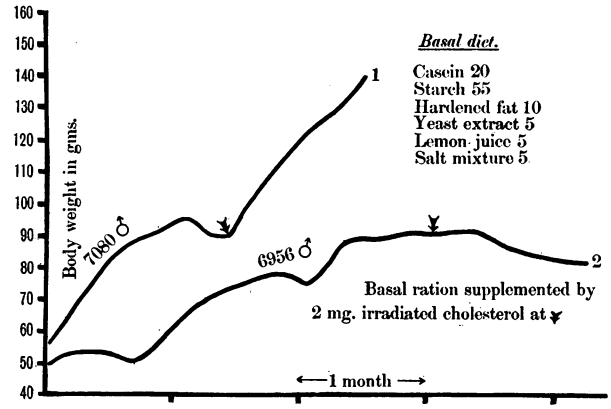

Fig. 1. Typical curves illustrating the two extreme types of response to an administration of 2 mg. of irradiated cholesterol daily. Curve 1 shows the prompt resumption of growth usually shown by animals which have previously shown the characteristic failure of growth at 4–5 weeks on the basal ration. Curve 2 shows failure of the irradiated cholesterol to cause resumption of growth in an animal that has been maintained on the deficient basal ration for 3 months.

The significant irregularities encountered were of two types. In some cases, illustrated by curve 2 in Fig. 1, the addition of the irradiated cholesterol produced no resumption of growth, but occasionally appeared to postpone the final decline. An analysis of the cases in this group revealed the interesting fact that practically all the animals that failed to respond had been maintained on the basal ration for a relatively long time; in the case illustrated by curve 2 (Fig. 1) the rat had been on the basal diet for three months before receiving the supplement of irradiated cholesterol.

The second type of irregularity that aroused our suspicion was shown by animals such as those whose growth curves are illustrated in Fig. 2. In these cases the resumption of growth on giving the irradiated cholesterol was prompt, and the rate sometimes almost normal, but after about four weeks a retardation set in followed by a rather rapid fall in weight. The important point, to our minds, was that the recovery we have described did not appear to be correlated with the dose of irradiated cholesterol given. This was indicated by the fact that daily supplements of 1–10 mg. appeared equally efficient in causing recovery, but, more particularly, by the fact that increasing many-fold the amount of the supplement after the second cessation (Y) of weight had occurred did not in a single case produce a further stimulation of growth (curve 4, Fig. 2). If, however, a small amount of cod-liver oil, or of a fraction rich in vitamin A prepared from cod-liver oil, was

administered at that point, a prompt and steady increase of weight again occurred (curve 5, Fig. 2). From this it may reasonably be concluded that the irradiated sterol was not supplying the substance known as vitamin A, and that the temporary development that followed the administration of this substance must have been due to another cause. To attempt an explanation of those results is, perhaps, unwise, until much more has been achieved in the experimental field, but provisionally the hypothesis tentatively advanced by Steenbock may be accepted, since it is not at variance with any of the observations we have recorded. The idea that the initial cessation of growth may be brought about by a deficiency of vitamin D in the basal diet at a time when the animal has not expended the reserves of vitamin A in its own tissues is one that fits in not only with the temporary response in growth that may follow the administration of the antirachitic vitamin D (irradiated cholesterol), but also with the fact frequently observed by us, that after a long period of maintenance on the basal ration animals usually fail to show any response to this substance; the long period of deprivation having, presumably, completely exhausted their reserves of vitamin A (curve 2, Fig. 1).

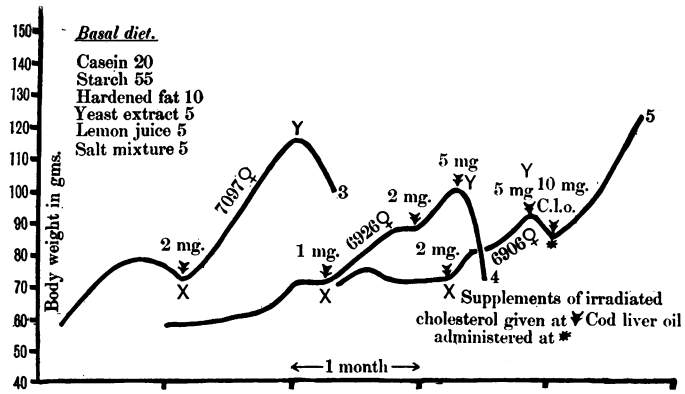

Fig. 2. Typical curves illustrating (3) rather rapid cessation of growth and decline (*Y*) after temporary resumption of growth on irradiated cholesterol (*X*). Curve 4 illustrates failure of growth in spite of increasing dose of irradiated cholesterol. Curve 5 illustrates power of cod-liver oil to prevent this decline.

There can be no question that Steenbock is right in emphasising the very complicated nature of the inter-relationships that exist between the many factors that determine growth in the young animal. Viewed from the standpoint of the value of tests for vitamin A the results of these observations are of considerable importance. In the past we have regarded the power to induce steady growth for a period of not less than three or four weeks as evidence that the substance, added as a supplement to the basal ration, contained the vitamin A. Judged by this criterion we should conclude that the irradiation of cholesterol by ultra-violet radiations synthesises a substance identical with, or possessing similar physiological action to vitamin A.

Obviously, however, unless the animals are protected throughout the test from a deficiency of the antirachitic substance, the method will be open to grave error. Indeed, we fear that no small proportion of the results that have been published on the occurrence and properties of vitamin A may have to be revised by tests in which this precaution has been taken.

We have referred to the means suggested by Steenbock for modifying the animal test for vitamin A. We have found it more convenient to employ irradiated cholesterol as a source of vitamin D in the basal diet. We usually prepare the "activated" material by radiation of either thin films of carefully purified cholesterol spread over the bottom of shallow flat dishes, exposed to air, or a thin film spread on the inner surface of a spherical quartz flask, evacuated by a mercury pump, and slowly rotated mechanically so as to ensure equal exposure of the whole surface. We are inclined to believe that the second method is preferable. In both cases we employ a Cooper-Hewitt quartz mercury-vapour lamp (220 v., 7·5 amp.) at a distance of 25 cm. for 2 hours, as a source of ultra-violet rays[1].

The animals are fed on a basal ration similar to that used by us for some years past, but from which we now exclude all fat. The mixture is compounded as follows:

Purified caseinogen	...	...	15 parts
Pure rice starch ...	...	...	70 ,,
Yeast extract ...	...	...	5 ,,
Salt mixture ...	...	...	5 ,,
Lemon juice ...	...	...	5 ,,

In a large number of experiments we have found that the exclusion of fat does not apparently disturb the normal behaviour of the animals on the basal ration[2]. As this is so, and as we have suspected that slight differences in the nature of the fats employed have at times been responsible for abnormal results, we decided to exclude them as being unnecessary potential sources of error.

The basal diet given above is, however, supplemented from the beginning of the experiment with 1·0 mg. of irradiated cholesterol daily, this being administered to the animals separately in the form of one drop of a solution in liquid paraffin. It may also be incorporated in the diet to the extent of 20 mg. %, so that each rat obtains 2–3 mg. daily.

[1] We are indebted to Prof. E. C. C. Baly, F.R.S., for much advice in connection with the use of these lamps. Too frequently it is not borne in mind that they exhibit "decay," that is that the proportion of radiations of short wave-lengths falls off with use. The "decay" would appear to be due to changes in the transparency of the silica walls of the lamp, by which they become more opaque to the shorter rays. This occurs more readily if the lamp is run "cold" at a low voltage, and, apparently, within limits, the hotter the lamp becomes when working the longer will it retain its transparency to the physiologically active rays. A rough test, that saves the trouble of periodic examinations of the light by optical methods, is the formation of ozone. As long as the smell of this gas is marked it is certain that the lamp is emitting rays that are in the part of the spectrum in which occur those responsible for the antirachitic effect.

[2] The successful rearing of rats for several generations on diets devoid of fats has already been reported [Drummond and Coward, 1921].

Typical growth curves of animals during this preliminary period are illustrated in Fig. 3 from which it will be seen that the initial growth is considerably greater than is shown by similar groups not receiving the irradiated cholesterol. Instead of the characteristic retardation of growth appearing at about the fourth week, when the animal weighs about 90 g., the supplemented basal ration enables the animals to grow for 5 to 6 weeks, reaching a weight of about 110–130 g. before their growth stops. Further maintenance on the basal diet, although enriched by the antirachitic factor, results in decline and death, and, as far as we have been able to ascertain, this end can only be prevented, and growth re-established, if a supply of the vitamin A is promptly administered[1].

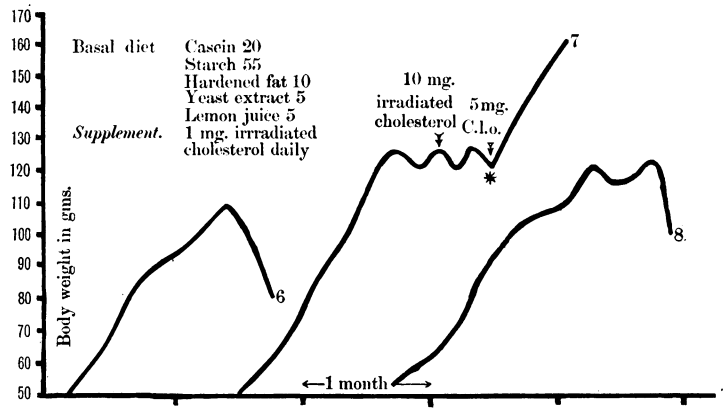

Fig. 3. Curves 6 and 8 show typical behaviour of rats fed on basal diet containing no fat and supplemented with 1 mg. irradiated cholesterol daily. The onset of the decline is sharp. Curve 7 shows failure of extra irradiated cholesterol to induce further growth, but prompt recovery on adding 5 mg. cod-liver oil.

It is interesting to note that these curves are rather sharply "peaked." The decline sets in almost as soon as the rats cease growing, and there is seldom a period of maintained body weight as there usually is on the old basal diet (Fig. 1).

It is unfortunate that the modification of the technique which we have described increases the time required to carry through a test to detect the presence of vitamin A in a substance.

[1] The administration of substances, in particular those relatively rich in vitamin A, to animals during these tests presents some difficulty. It is useless, in our opinion, to incorporate them with the diet. Zilva and Miura [1921, 1, 2] introduced the use of olive oil aerated for many hours at 100° to destroy vitamin A, as a diluent, and administered measured drops of a solution in this medium of the substance to be tested. Even this procedure, which was adopted by us for some time, appears to be open to criticism, because the aeration of the olive oil results in the formation of substances, loosely termed peroxides, which appear to have a destructive action on the vitamin A present in the substance to be tested [Fridericia, 1925].

We have more recently employed liquid paraffin (medicinal) as a diluent. This has the advantage of being free from vitamin A, and of being an inert solvent suitable for use with fatty substances. On the other hand we are not yet satisfied that absorption of substances dissolved in this medium is quantitative.

It is for this reason that we are concentrating our attention on methods which will replace the expensive and time-consuming feeding experiments with animals. In a recent communication [Rosenheim and Drummond, 1925] we have outlined colour reactions which we believe are given by vitamin A, and which we hope will before long be made the basis of methods of quantitative estimation. We have in the last month or two attempted to compare the sensitivity of these colour reactions with that of the rat-feeding tests. It has proved a harder task than we anticipated, and we do not wish to commit ourselves to a definite opinion until further work has been completed. It may be said, however, that the sensitivity of the colour reactions with trichloroacetic acid or dimethyl sulphate, as determined by their value in comparing the vitamin A content of different samples of cod-liver oil, or different dilutions of the same sample of cod-liver oil in a neutral solvent, seems to be of about the same order as the animal test. The reaction with arsenic trichloride is decidedly more delicate.

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